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Technical approaches to inoculate micropropagated sugar cane plants were *Acetobacter diazotrophicus*

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Abstract

Micropropagated plantlets of sugar cane were inoculated with the N_2-fixing bacterium *Acetobacter diazotrophicus*. Various modifications on the basic plant culture medium MS were made for the plant/bacteria association. The protocol required the inoculation of the bacteria at the end of the rooting period in a medium without hormones or vitamins, and with the concentration of sugar and mineral nutrients reduced by a factor of 10. Individual plants were inoculated with *A. diazotrophicus* and maintained under the appropriate light and temperature condition used for micropropagation up to 7 days. The system favored the infection and the establishment of the bacteria within the plant tissue. Bacteria colonized the plant tissue and accumulated in inter-cellular cavities and the region of lateral root emergence and also colonizes the xylem vessels. The inoculated plantlets were subsequently transferred to the acclimatization phase and after 30 days it was possible to isolate the bacteria from plant tissue. This protocol permitted studies of infection and comparison among strains.

Abbreviations: BNF – Biological Nitrogen Fixation

Introduction

In Brazil, many sugar cane farmers are using micropropagated sugar cane varieties in nurseries. This technique allows them to obtain plant material free from pathogenic bacteria and viruses. Unfortunately, this protocol suppresses the nitrogen-fixing bacteria, especially those endophytic bacteria like *Acetobacter diazotrophicus* and *Herbaspirillum* spp., all found in abundance within sugar cane. Some of the Brazilian sugar cane varieties have been shown to obtain more than 60% of their nitrogen from BNF (Lima et al., 1987; Urquiaga et al., 1992). The beneficial aspects of the endophytic localization of these bacteria was recently revised by James and Olivares (1998).

A. diazotrophicus was originally isolated from roots and stems of sugar cane (Cavalcante and Dobereiner, 1988), and it has several peculiar characteristics, such as growth tolerance to 10% sucrose and initial pH 5.5, nitrogen fixation even at pH below 3.0, no use of NO_3^- and nitrogenase activity partially inhibited only by the presence of NH_4^+ especially in a medium with high sugar concentration (Reis et al., 1990; Stephan et al., 1991). Ecological studies of this organism have shown that *A. diazotrophicus* do not survive in the soil or in weed species found in cane fields (Baldani et al., 1996; Li and MacRae, 1992; Reis, 1996). However, it has been found in high numbers in sugar cane roots, stems and in green and dry leaves and in the trash. Because *A. diazotrophicus* is not able to survive outside of the plant tissue, it is mainly transferred to subsequent crops via setts (stem pieces) used in the vegetative propagation of sugar cane (Reis et al., 1994). The use of monoxenic micropropagated sugar cane plantlets may eliminate this mode of bacterial transfer. Although no evidence exists, it is possible that other ways of infection could reintroduce this bacterium into the plant tissue such as the remained of the root material, the trash, or infection by mealybugs where these endophyte have been detected.

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Recently, it was shown that the heat treatment (50 °C for 150 min) of the setts, routinely used to control the pathogen of ratoon stunting disease (*Clavibacter xylii* subsp. *xyli*), had no effect on the nitrogenase (acetylene reduction) activity of the sugar cane plants (Reis et al., 1994). This suggests that it is not possible to obtain setts free from N_2-fixing bacteria to be used as a control in inoculation experiments. On the other hand, monoxenic micropropagated plants can facilitate the inoculation of sugar cane with selected N_2-fixing endophyte bacteria, and therefore allow the introduction of either non-modified or genetically engineered bacteria.

In this paper we explore the potential of this technique and compare the performance of *A. diazotrophicus* strains as well as varieties of sugar cane under monoxenic cultures and during the acclimatisation period of the transplanted plantlets.

Materials and methods

Organisms, growth conditions and bacterial inoculation

Sugar cane (*Saccharum* spp.) varieties NA56-79, SP70-1143, CB45-3, B-4362, SP79-1011 and SP71-6163 were micropropagated according to a method described by Hendre et al. (1983) using the apical meristem. This methodology utilizes the MS medium (Murashige and Skoog, 1962) modified in respect to the hormone concentration to promote the callus multiplication (phase I), multiplication of aerial part (phase II) and roots (phase III) during 80 to 90 days. They were then separated and single plants were selected by size and transferred to 50 mL of a modified MS medium without hormones or vitamins. The concentration of nutrients and sucrose in the medium was altered according to individual experiments.

A. diazotrophicus strains PAL-5 (ATCC 49032) and PSP-32 (Cojho et al., 1993) were grown in Dygs medium (Rodrigues Neto et al., 1986) containing (g per liter): glucose, 2.0; glutamic acid, 1.5; peptone, 1.5; K_2HPO_4, 0.5; $mgSO_4.7H_2O$, 0.5; yeast extract, 2.0; pH 6.0 for 24 h. Plantlets were inoculated with 0.1 mL of suspensions containing 10^6 to 10^7 cells per mL. Controls were inoculated with autoclaved bacteria. Plants were maintained at 30 °C with 60 μmol m^{-2} s^{-2} illumination for 12 h day^{-1}.

Counting of *A. diazotrophicus* was performed using serial dilutions in 5% sucrose solution followed by inoculation into selective semi-solid N-free LGI-P medium (Reis et al., 1994).

'In vitro' assays

Experiment 1. Effect of sucrose and nitrogen sources
Plantlets of varieties NA56-79 and SP70-1143 were inoculated with strain PAL-5. The treatments using modified MS medium were as follows: (A) control – complete MS medium with 20 g/L of sucrose, (B) MS medium without sucrose. Plantlets were harvested at 15 and 21 days after inoculation with 3 replicates. In the last harvest, after weighing the fresh material, the entire plants were surface sterilized with chloramine T 1% for 5 min, washed in buffer and water, blended and the bacterial cells were counted. The pH of the medium was also measured.

Experiment 2. Effect of sucrose and nitrate concentration
Variety SP70-1143 was inoculated with *A. diazotrophicus* strains PAL-5 and PSP-32. The sucrose concentration in the MS medium was decreased from 20 g $liter^{-1}$ to 2 g $liter^{-1}$ and KNO_3 was used as a sole nitrogen source in accordance to the treatments: (1) 20 m *M*, (2) 7 m *M*, and (3) 3 m *M*. Plants were analysed for fresh weight 15 days after inoculation.

Experiment 3. Effect of sugar cane varieties and medium compounds
Varieties NA56-79, SP70-1143, B4362, CB 45-3 and SP79-1011 were inoculated with strain PAL-5. The concentration of salts and sucrose of the MS medium was reduced to 1/10 of the concentration routinely used in the standard MS medium. Plants were harvested after a 7-day incubation period and the entire plant was superficially sterilized with chloramine T 1% using different incubation times. Plant material was divided for bacterial counts (1 g of fresh weight) and the variety SP70-1143 was used for microscopical analysis. Small pieces of roots and aerial parts were cut (2–5 mm) and fixed in 5% glutaraldehyde in 50 m *M* phosphate buffer (pH 7.0) for 24 h and the material was examined under the scanning electron microscope (SEM). For this purpose, samples were dehydrated in an ethanol series and gradually substituted by acetone. The specimens were critically point-dried in a Biorad E 3000 critical point dryer and subsequently coated with gold in a Biorad E5 200 auto sputter coater. Specimens were viewed on a Cambridge Stereoscan 200 SEM

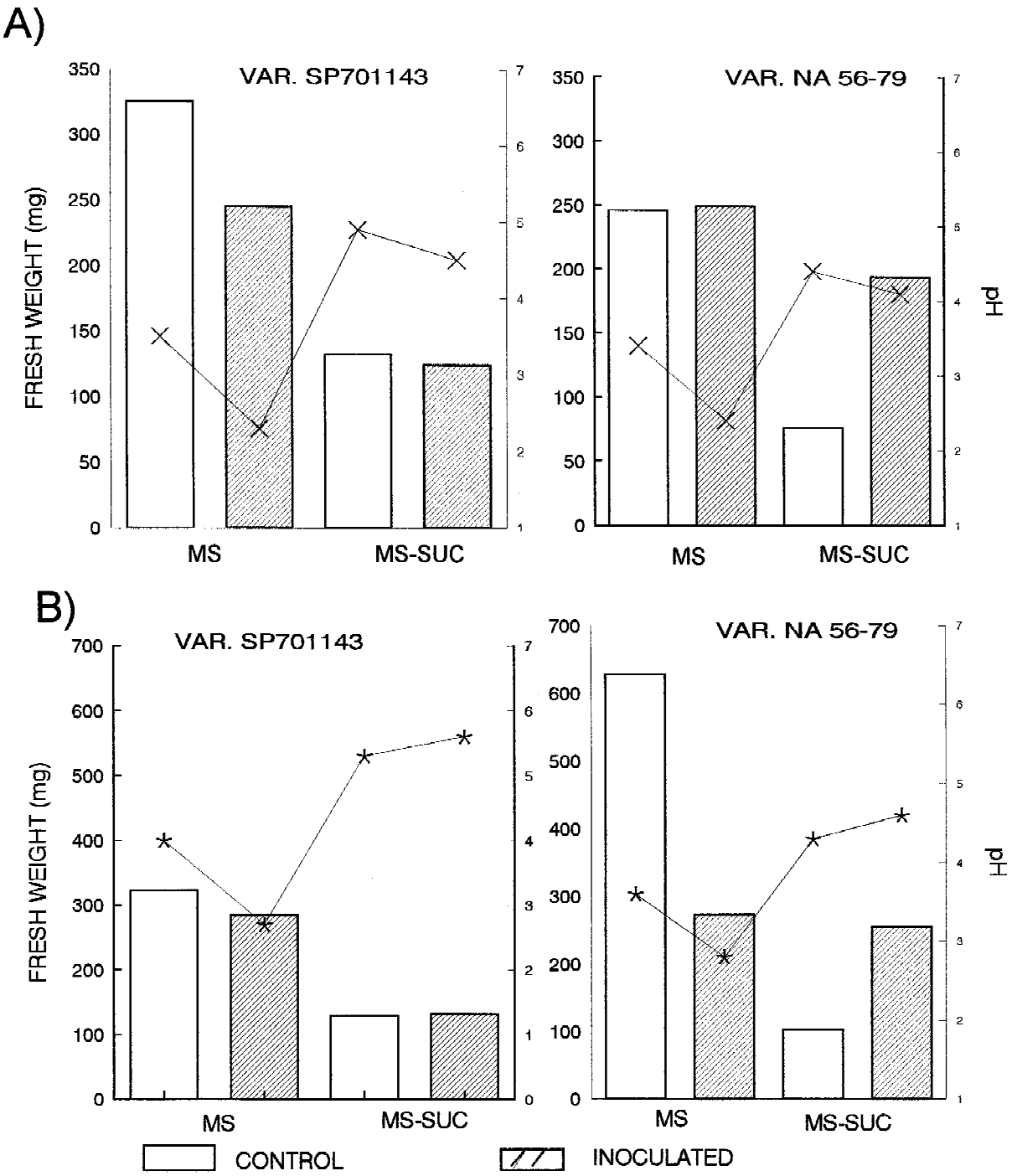

Figure 1. Effect of inoculation with *A. diazotrophicus* strain PAL_5 on fresh weight of two sugar cane varieties harvested 15 (A) and 21 (B) days after inoculation. MS - Complete medium with sucrose (20 g/L) and MS-SUC: Without sucrose.

Acclimatization assays

Sugar cane varieties SP70-1143 and SP71-6163 were inoculated '*in vitro*' with *A. diazotrophicus* strains PAL-5 and PSP-32. Seven-day-old plants were transferred to polystyrene trays containing sand, vermiculite and peat (2:1:1/2) and maintained in controlled conditions for 30 days in the greenhouse. Three sam-

ples were harvested to measure the number of *A. diazotrophicus* cells. Another three samples were cut into pieces of 1–2 mm from the root to the tops at intervals of 5 cm and inoculated into LGI-P medium.

Statistical analysis

All tests were performed using MSTATC program and media were compared by Tuckey $p < 1.0\%$.

Results and discussion

In order to optimise simultaneously growth of both plantlets and N_2-fixing bacteria, the traditional MS medium was modified. The presence of nitrogen and a high amount of sucrose in the medium promoted an abundant growth of *A. diazotrophicus* which was detrimental to the plantlets. Thus, it was necessary to develop a medium where the bacterium would be dependent on the presence of the plant to survive and maintain the N status enough to keep the nitrogenase activity derepressed.

Plant response to *A. diazotrophicus* inoculation was variable after 15 (Figure 1a) and 21 days (Figure 1b) after inoculation. The removal of sucrose from the medium partially inhibited growth of the plantlets of both varieties. During this period, light induces the formation of chlorophyll (Edelman and Hanson, 1971; Yamada and Sato, 1978) but the plants could not perform photosynthesis for their normal growth and therefore were dependent on the sucrose content. In a complete MS medium, high numbers of *A. diazotrophicus* negatively influenced the growth of variety NA56-79, the variety which had shown the best growth (Figure 1a and b). The decrease of pH level after inoculation could explain this negative effect (Figure 1a and b). This effect was not apparent when the bacteria were dependent only on plant-derived carbon sources (Figure 1a and b). Even when the plants were negatively influenced by the absence of sucrose, the bacteria were viable on the tissue in numbers higher than 10^6 cells g^{-1} fresh weight (data not shown). Plants ceased their growth in sucrose-free medium 21 days after inoculation (Figure 1 b). In the absence of sucrose, the pH of the medium increased in comparison with the non-inoculated controls. Plant growth was drastically reduced and showed severe chlorosis when the pH increased above 6.0. Nevertheless, the variety SP70-1143 showed less change in the pH of the solution.

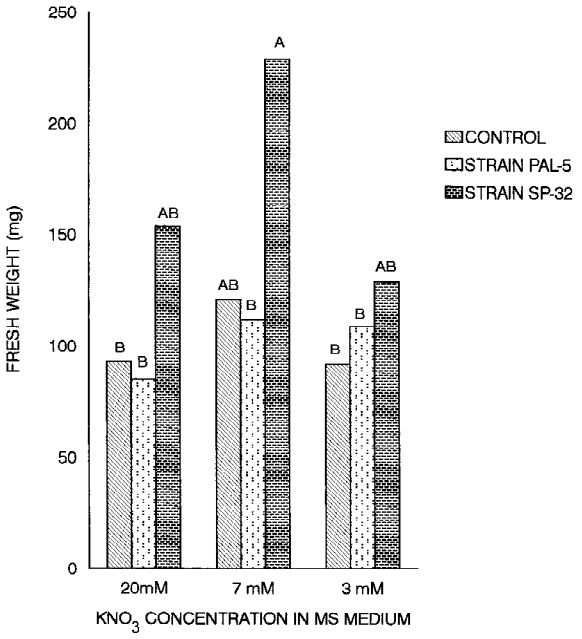

Figure 2. Effect of the inoculation of two *A. diazotrophicus* strains and level of nitrate on the fresh weight of micropropagated sugar cane plants harvested 15 days after inoculation.

The reduction of sucrose concentration to 2 g L^{-1} and use of NO_3^- as the nitrogen source was also tested. Because *A. diazotrophicus* does not possess nitrate reductase and therefore is not able to use NO_3^- it would be expected that this N source could be used only by the plant. Under these conditions, inoculation with strain PSP-32 (isolated from washed, crushed stems of sugar cane from São Paulo State) showed a greater increase in plant fresh weight than strain PAL-5 (isolate from sugar cane roots from Alagoas State) (Figure 2). It is possible that strain PSP-32 is better adapted to the variety SP70-1143 which is a genotype bred by COPERSUCAR in the state of São Paulo. The significant response of variety SP70-1143 in the presence of the strain PSP-32 was higher in the medium with 7 mm NO_3^- (Figure 2).

Based on these data, the MS medium was again modified and tested. The concentration of all salts and sucrose was reduced by a factor of 10 to maintain the ionic balance, since the original medium contained high salt concentration to support plant growth for much longer periods (25–30 days). The data on plant infection showed that after 7 days, the number of *A. diazotrophicus* in roots and leaves was higher than 10^6 cells g fresh weight^{-1} and this internal popula-

Table 1. Counting of *A. diazotrophicus* population in micropropagated sugar cane plantlets 7 days after inoculation (number of cells . g^{-1} fresh weight)

Sugar cane varieties	Surface sterilization with chloramine-T 1% (a)		
	1 min	5 min	10 min
Control	0	0	0
NA56-79	$> 1.4\times10^7$*	1.4×10^7	1.4×10^7
SP70-1143	$> 1.4\times10^7$	1.5×10^7	1.4×10^7
B-4362	$> 1.4\times10^7$	n. d.	1.4×10^7
CB45-3	$> 1.4\times10^7$	n. d.	1.4×10^7
SP79-1011	$> 1.4\times10^7$	n. d.	1.4×10^7

(a) Means of 3 replicates samples.
* Numbers are superior than the limit of dilutions utilized.
n.d. = not determined.

tion could still be quantified after surface sterilisation in chloramine T (1%) for 5–10 min. (Table 1). These results showed that a shorter time of incubation could be used to provide good bacterial colonization of the plant tissue. The colonization of the plant was analysed 4 and 7 days after inoculation onwards to select the best incubation time. It was possible to localise the bacteria on the plant surface under the SEM and the number of *A. diazotrophicus* cells increased with time (James et al., 1994). *A. diazotrophicus* grew quickly on the surface of the plant tissue and these numbers were maintained with time (up to 10^6cell ml^{-1} data not shown). For these reasons, the incubation time could be shortened to 7 days without affecting the infection and the resulting plants were ready to be transferred to the acclimatisation process.

By testing different varieties and time of sterilisation it was demonstrated that *A. diazotrophicus* were present in high numbers and that the sterilisation procedure did not decrease the bacterial number inside the tissue (Table 1). This result could be explained by the high numbers of bacteria present in the cracks of the root tissue (Figure 3a) and in the region of the emergence of secondary roots as shown by James et al. (1994) using this technique. The localization and also the high numbers of cells found could explain the requirement of a longer surface sterilization periods (5–10 min with chloramine T 1%) to decrease the numbers of bacteria present in the plant (Table 1). Our results showed that *A. diazotrophicus* cells were randomly distributed on the plant surface in an apolar orientation forming a monolayer covering roots and leaves (Figure 3b). The lateral root junction showed a high number of *A. diazotrophicus* cells (Figure 3c) and also colonizes the xylem vessels (Figure 3d).

The ability of *A. diazotrophicus* cells to survive in association with the plants during the acclimatisation period was evaluated using a mixed substrate with a low nutrient concentration to induce the plant to respond to inoculation. The absence of carbon substrate during the acclimatisation period induced photosynthesis in the plants and it was also necessary that the root system grew quickly to take up nutrients from the solution since the root biomass was insignificant during the '*in vitro*' period. These modifications were found to diminish the bacterial population associated with the plants (Table 2). Examination of the plant tissue for the presence of *A. diazotrophicus* cells showed that this bacterium remained on older root and shoot tissues but not in the substrate (Table 3).

The results of this study indicate an alternative way to inoculate micropropagated sugar cane plants with *Acetobacter diazotrophicus*. This bacterium does not possess an active mechanism to penetrate directly in the plant tissue and the internal tissue colonization probably occurred by natural openings or wounds provoked by the individualisation process. Also it was shown by James et al. (1994) that the bacteria infect the plant tissue via the lateral root junctions between wounds in the epidermal cells or through the root tips. In a previous study, it was found that inoculation with bacteria on sugar cane plants grown in soil did not allow infection of the plants, except in the presence of arbuscular mycorrhizal fungi (Paula et al., 1991). The technique developed here for '*in vitro*' inoculation resulted in an abundant infection the plant by *A. diazotrophicus*. The advantage of this technique is that a single strain of *A. diazotrophicus*, either wild type or genetically modified, can be established in monoxenic plants. The system can also be used to study the

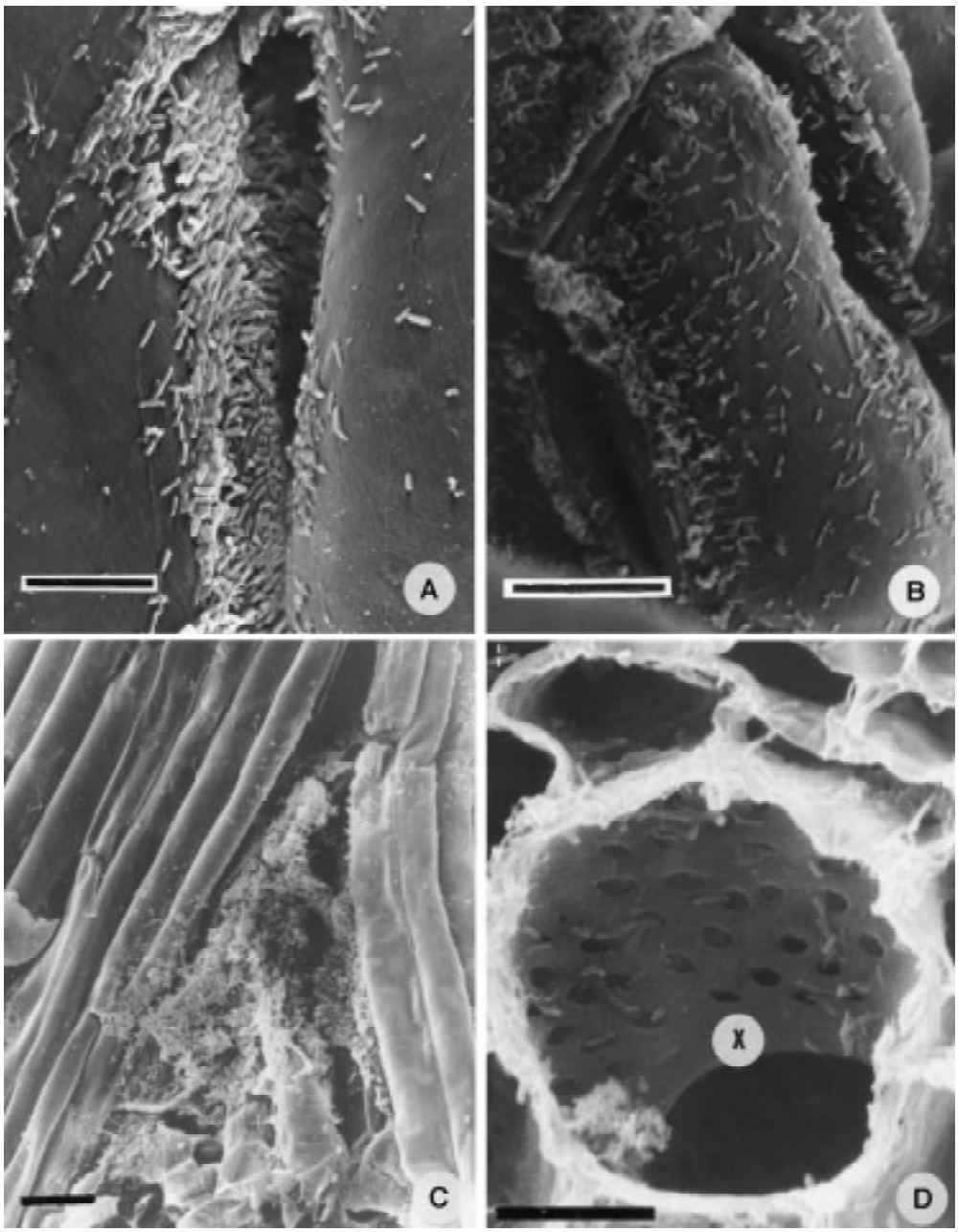

Figure 3. (*A*) Scanning electron micrograph showing *Acetobacter diazotrophicus* strain PAL-5 colonizing a crack entrance between two epidermal cells of sugar cane root var. SP70-1143, 7 days after inoculation (bar = 10μm). (*B*) Scanning electron micrograph showing *Acetobacter diazotrophicus* strain PAL-5 attached to the epidermal cells of the bottom of the sugar cane stems var. SP70-1143, 7 days after inoculation (bar = 10μm). (*C*) Scanning electron micrograph showing *Acetobacter diazotrophicus* strain PAL-5 colonizing a crack entrance of sugar cane var. SP70-1143, formed at the site of a lateral root junction (bar = 25μm). (*D*) Scanning electron micrograph of a xylem vessel of sugar cane stem var. SP70-1143 colonized by *Acetobacter diazotrophicus* strain PAL-5 (bar = 10μm).

interaction with other organisms, e.g. *Herbaspirillum* spp. Further studies are underway in pots and in the field to evaluate the plant response to inoculation with *A. diazotrophicus* and to quantify the contribution of plant nitrogen derived from N_2 fixation.

Table 2. Enumeration of *A. diazotrophicus* population in roots and aerial part of sugar cane plants 30 days after acclimatization in solid substrate

Varieties	N° of cells.g^{-1} fresh weight ($\times 10^4$)					
	Roots			Aerial Parts		
	Control	PSP-32	PAL-5	Control	PSP-32	PAL-5
SP70-1143	0[a]	4.5	2.5	0	30.0	4.0
SP71-6163	0	0.2	0.2	0	54.0	4.4

[a] means of 3 replicates. Estimation by the Most Probable Number (MPN).

Table 3. Reisolation of *A. diazotrophicus* from sugar cane plants varieties SP70-1143 and SP71-6163 after the acclimatization period

Plant tissue	Growth on LGI-P medium		
	Control	PSP-32	PAL-5
Root tips	–	–	–
Upper roots	–	+	+
Basal stems	–	+	+
Old leaves	–	+	+
New leaves	–	–	–
Senescent leaves	–	+	+
Substrate	–	–	–

+, Positive growth; –, negative growth. Tree vials of semi-solid LGI-P medium was used for each plant part.

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